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Efficient β -Cell Regeneration by a Combination of Neogenesis and Replication Following β -Cell Ablation and Reversal of Pancreatic Duct Ligation

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Key Words. diabetes mellitus • beta-cells • neogenesis • replication • regeneration • islet of Langerhans

ABSTRACT

Achieving efficient β -cell regeneration is a major goal of diabetes research. Previously, we found that a combination of β -cell ablation and pancreatic duct ligation led to β -cell regeneration by direct conversion from α -cells. Here, we studied the effect of surgical reversal of the duct ligation, finding that there was a wave of β -cell replication following reversal. The combination of β -cell neogenesis prior to reversal of the duct ligation and β -cell replication following reversal

resulted in efficient β -cell regeneration and eventual recovery of function. This provides an important proof of principle that efficient β -cell regeneration is possible, even from a starting point of profound β -cell ablation. This has important implications for efforts to promote β -cell regeneration.

INTRODUCTION

Because decreased β -cell number is a central feature of both type I and type II diabetes, inducing β -cell regeneration has been a focus of efforts to develop a definitive treatment for diabetes. A prevalent approach to studying β -cell regeneration is to induce damage to the pancreas and then dissect the process by which regeneration occurs. Common damage models have included partial pancreatectomy [1, 2],

pancreatic duct ligation (PDL [3-5]), and chemical β -cell ablation [4, 6].

An issue of considerable importance that has been inadequately addressed is whether β -cell regeneration can fully reconstitute a normal number of β -cells, particularly starting from a condition of severe β -cell deficiency as occurs in type I diabetes. In large part, efforts to address that question have been confounded by the considerable controversy that exists about the mechanism and the extent to which β -cell

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regeneration occurs in the various damage models [1, 4, 5, 7, 8]. In fact, most damage models, including PDL and partial pancreatectomy, do not involve β -cell ablation, and in most studies in which β -cell ablation has been a feature, it has not been efficient, making it impossible to determine the maximum extent of β -cell regeneration from the very low starting point that exists in some cases, particularly type I diabetes. Efficient β -cell ablation using a transgenic mouse in which the diphtheria toxin receptor was expressed under the control of the insulin promoter has been studied, with the finding that β -cell neogenesis from α -cells occurred, but only partial β -cell reconstitution was achieved and reversion to normoglycemia took 5-10 months [9].

Previously, we developed a new model of pancreatic damage that combined PDL and severe β -cell ablation using high dose alloxan [4]. While it is now clear that β -cell neogenesis from duct cells or Ngn3-positive intermediates does not occur following PDL alone [5, 8], when we combined PDL with β -cell ablation, β -cell neogenesis from α -cells was rapid and efficient. α - and β -cell replication occurred to some extent, but neogenesis and replication were completely unlinked, consistent with their being under distinct regulatory control. Because of the severe and continuing injury from PDL, it was not possible to maintain the mice for a long enough period to study the long term outcome. In particular, we could not determine the full extent of recovery in the number of β -cells or the extent to which the increased number of β -cells would translate into increased β -cell function.

We hypothesized that reversing the effects of the inflammatory milieu in which the neogenic β -cells existed following PDL might allow us to answer questions about the extent of β -cell recovery following PDL plus alloxan. One possibility was that β -cell regeneration would cease once the damage stimulus of PDL ended. Another possibility was that β -cell

regeneration, whether in terms of β -cell number or maturation, would continue. To distinguish between those possibilities, we surgically reversed the PDL. PDL reversal (Re-PDL) resulted in rapid normalization of the exocrine pancreatic architecture and allowed us to follow the mice for a longer period to determine the ultimate effect on β -cell number and function.

Following Re-PDL, the number of β -cells increased enormously. Surprisingly, this was due to a wave of β -cell replication. Over a long period, newly formed immature β -cells acquired characteristics of mature β -cells, including the expression of MafA, membrane-localized GLUT2, and PDX-1. Islet architecture, which was severely disrupted following PDL plus alloxan, gradually reverted to the normal state, with β -cells internal to α -cells. The combination of β -cell neogenesis following PDL plus alloxan in combination with the β -cell replication that occurred following Re-PDL resulted in a normal number of β -cells. We conclude that, at least in young mice, β -cell regeneration from a starting point of profound β -cell deficiency is sufficiently robust to reconstitute a normal number of β -cells and lead to a major improvement in β -cell function. This finding offers encouragement for efforts to induce effective and efficient β -cell regeneration.

MATERIALS AND METHODS

Animal procedures

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Sanford-Burnham Medical Research Institute in accordance with national regulations.

PDL and PDL Reversal

Four week old male ICR mice were purchased from Harlan Sprague Dawley. All Re-PDL procedures were done with ICR mice, in contrast with our previous study [4] that used

primarily C57BL/6J mice. The choice of ICR mice was dictated by the fact that Re-PDL was technically difficult and associated with high mortality. ICR mice are larger than C57BL/6J at the same age (26-30 grams versus 22-25 grams at 4 weeks of age) and gain body weight faster under normal conditions and postoperatively. On average, mice lost approximately 1/3 of their body weight in the postoperative period, making it advantageous to use larger mice to start. In terms of the surgical procedure, we found that C57BL/6J had more connective tissue between the abdominal organs than ICR mice, making surgery on C57BL/6J more difficult.

Mice underwent PDL plus alloxan treatment as previously described [4, 10]. One week following PDL plus alloxan, mice with blood glucose levels $>500\text{mg/dL}$ were divided into 2 groups: PDL ($n=10$) and PDL followed one week later by Re-PDL ($n=26$). Six of the 10 PDL alone mice died within two weeks of surgery. Pancreases of sufficient quality for analysis were harvested from two mice that died or became sick and so were sacrificed per the terms of our animal care protocol at 17 and 19 days following PDL plus alloxan injection-analyzed as the PDL alone early group. Two PDL alone mice survived for 35 and 52 days following PDL and were analyzed as the PDL alone late group.

Of the 26 mice that survived for at least one week following Re-PDL, 5 had tissue harvested between days 15 and 18 following PDL and were analyzed as the Re-PDL early group. Six Re-PDL mice had tissue harvested at late times following PDL and were analyzed as the Re-PDL late group. One mouse in the Re-PDL group exhibited partial recovery of the exocrine pancreas and was analyzed separately. The pancreases from the remaining mice could not be harvested due to degradation. The day of sacrifice for the mice in the late groups is presented in the figure legends.

Re-PDL was done as follows: After anesthesia with ketamine/xylazine, sutures that were used to close the skin and abdominal muscles were removed and the incision was opened by gently stretching skin and abdominal muscle on both sides. The pancreas distal to the ligation exhibited severe exocrine atrophy, with islets being visible surrounded by translucent connective tissue and blood vessels. The suture used to ligate the duct was removed by gently untying the knot. The duct distal to the ligation was gently massaged until fluid was observed flowing antegrade through the duct. The wound was then closed.

To determine which cells replicated following PDL or Re-PDL, BrdU (1mg/ml, Sigma-Aldrich) was administered in the drinking water beginning on day 1 after Re-PDL or day 8 after PDL and continuing for 5-6 weeks. Diabetic mice were injected subcutaneously once daily with insulin glargine (Sanofi-Aventis). Insulin doses were adjusted depending on blood glucose level. After the mice had 4 consecutive blood glucose measurements $<200\text{mg/dL}$ within one week, an intravenous glucose tolerance test (IVGTT) was performed to test for β cell function. After IVGTT, the mice were sacrificed and the pancreas harvested. When the pancreas was harvested for histology in the Re-PDL group, the section of pancreas distal to the ligation had greatly increased in size but still appeared to be slightly smaller than prior to PDL. Compared with the proximal pancreas, it was whiter in color, allowing it to be clearly distinguished from the proximal pancreas. Using that morphological difference, the pancreas was separated into two parts, one proximal and one distal to the PDL.

IVGTT

For IVGTT, the mice were fasted for 12 hours, followed by penile vein injection of 0.5 g/kg Dextrose (Hospira, Lake Forest, IL). Blood was collected via tail vein at 0, 1, 5, 10, 15, 30, 60, 90, 120, 150 and 180 minutes after dextrose injection. A small drop of blood was withdrawn

from the tail vein and glucose measured using a OneTouch UltraMini glucometer.

Histology and Immunochemical staining

Tissue was fixed in 4% paraformaldehyde (Santa Cruz Biotechnology, Inc) for 12 hours at 4°C, washed in Phosphate buffered saline (PBS), followed by overnight incubation at 4°C in 30% sucrose (Sigma-Aldrich). Samples were then embedded in OCT compound and frozen at -80°C. Cryosections of 5 μ m thickness were incubated with primary antibody (Table 1) overnight at 4°C. Secondary antibodies (Table 1) were added and incubated for 45 minutes. Antigen retrieval was used for BrdU and MafA staining. Nuclei were visualized by DAPI staining (Sigma-Aldrich).

Quantification of β -cell area and number

All slides were scanned and analyzed using the Aperio ScanScope XT system (version 10, Aperio Technologies, Vista, California). Insulin positive area per section was calculated by dividing the area of insulin positive area by the pancreas area. The number of insulin positive cells was calculated by counting the number of DAPI-positive nuclei surrounded by insulin positive cytoplasm. The number of insulin-BrdU double-positive cells was calculated by counting the number of BrdU stained nuclei surrounded by an insulin-positive cytoplasm.

Statistical analysis

Data are presented as mean \pm SEM. The statistical significance of the differences between groups was analyzed by Student's t-test. The Kaplan-Meier curve was analyzed by log-rank test.

RESULTS

Reversal of PDL led to rapid recovery of the exocrine pancreas

PDL reversal (Re-PDL) was performed by untying the suture used to ligate the pancreatic duct one week after PDL plus alloxan. While PDL led to complete (compare Figure 1 A and

B) and irreversible (Figure 1D) elimination of the exocrine enzyme amylase expression, Re-PDL led to rapid (Figure 1F) and almost complete (Figure 1H) recovery of the exocrine pancreas (quantified in Figure 1M). In neither PDL nor Re-PDL animals was there any effect on the exocrine pancreas proximal to the ligation (Figure 1A, E, C, G).

To monitor for cellular replication following Re-PDL, BrdU was administered in the drinking water beginning one day after reversal (or the analogous time for the controls) and continuing for 5-6 weeks. Unexpectedly, the acinar cells present after Re-PDL were composed of a mixture of BrdU-positive and BrdU-negative cells. This indicates that many acinar cells did not arise by replication of an acinar cell precursor, but rather from pre-existing acinar cells that had lost amylase expression.

β -cell neogenesis following PDL and Re-PDL

Consistent with our previous study of β -cell neogenesis following PDL plus alloxan [4], large numbers of cells co-expressing α - and β -cell markers, most notably glucagon and insulin, were found within two to three weeks following PDL plus alloxan (yellow cells in Figure 2B, F, Suppl. Figure 1). There was almost no colocalization of insulin and glucagon at later times following PDL or Re-PDL (Figure 2D, H), indicating that β -cell neogenesis occurred in a brief wave.

Also consistent with our previous results, β -cell regeneration did not occur proximal to the ligation in either PDL or Re-PDL animals (Figure 2C, G)(Figure 3 C, G, K, O)(quantified in Figure 3R, S). This indicates that the stimulus for β -cell neogenesis was local, i.e., restricted to the pancreatic tail, the area distal to the PDL. This is in contrast to β -cell replication following partial pancreatectomy, where the effect of partial pancreatectomy on β -cell replication was equal throughout the

pancreas, regardless of the distance from the site of the surgery [1].

β -cell replication and normalization of β -cell area following Re-PDL

At later times following Re-PDL, there was a dramatic increase in the number of β -cells compared with PDL (Figure 2H vs. D)(Figure 3H vs. D, P vs. L, quantified in Q, R). Since there was no evidence of continuing β -cell neogenesis in the form of cells coexpressing multiple hormones, we examined β -cell replication as a possibility. Most of the new β -cells shortly after Re-PDL were negative for BrdU (Figure 3N, quantified in S), consistent with their having arisen by neogenesis rather than by replication of the rare β -cells that survived alloxan treatment. However, at late times following Re-PDL, there was an enormous increase in the number of BrdU-positive β -cells (Figure 3H, P, quantified in S). Thus, an unexpected wave of β -cell replication occurred following Re-PDL.

Islet structure and β -cell area returned to normal following Re-PDL

A conspicuous feature of islets immediately following β -cell ablation and regeneration is a disorganized structure in which the different islet cell types are intermingled rather than segregated with β -cell internal to the α - and δ -cells (Figure 4A, B). With the large increase in the number of β -cells following Re-PDL and the opportunity to follow the animals for a prolonged period, it was of interest to determine whether the islet structure returned to normal. In contrast with the disorganized structure early after PDL and Re-PDL, by 10 weeks following Re-PDL the islet structure had begun to normalize (Figure 4C), and essentially normal islets were found at later times (Figure 4D).

To determine the extent to which a combination of β -cell neogenesis and β -cell replication could reconstitute a normal number of β -cells, we measured the insulin-positive area as a fraction of the distal or proximal

pancreatic area on each section. Strikingly, the β -cell area following Re-PDL was indistinguishable from normal mice that had not undergone surgery or β -cell ablation (Figure 4E), demonstrating virtually complete β -cell regeneration from almost complete absence following high dose alloxan.

β -cells gradually matured following Re-PDL

Previously, we found that β -cells that were present following PDL plus alloxan had an immature phenotype [4]. This appeared to be due to selective survival of a preexisting population of immature cells that survive high dose alloxan due to the absence of GLUT2, which is required for alloxan to enter the cell [11]. Similar to our previous results [4], insulin-positive cells in the early period following PDL or Re-PDL lacked detectable levels of GLUT2, MafA, or PDX-1 (Figure 5). Following Re-PDL, the β -cells gradually matured, acquiring all three of those markers of functional β -cells (Figure 5H, P, X).

A mouse in which Re-PDL was only partially successful as determined by limited exocrine regeneration (Suppl. Figure 2A–C) was instructive. It failed to exhibit maturation of the surviving β -cells, which remained negative for PDX-1 (Suppl. Figure 2D, E), MafA (Suppl. Figure 2F, G), and GLUT2 (Suppl. Figure 2H, I). β -cell regeneration was restricted to areas where acinar regeneration took place (compare Suppl. Figure 2B and C, high power view of regenerating islets inset), suggesting that the pancreatic milieu following PDL is unfavorable for β -cell maturation.

Mice with Re-PDL exhibited improved glucose homeostasis and survival

Given the maturation of β -cells as determined by the acquisition of GLUT2, MafA, and PDX-1 in mice that underwent Re-PDL, we performed a time course analysis of the blood glucose concentration. As we found previously, the control PDL plus alloxan mice remained severely diabetic (red line in Figure 6A) and none survived past day 50 (red line in figure

6B). However, the blood glucose concentration in mice that underwent Re-PDL began to drop immediately following Re-PDL (black line in Figure 6A). Mice that underwent Re-PDL had a highly statistically significant survival advantage (Figure 6B). The mouse that had incomplete Re-PDL (Suppl. Figure 2) survived until day 150, but did not revert to normoglycemia (green line in Figure 6A). No long term surviving mice were found in which acinar cell recovery did not occur. To determine the extent to which β -cell function was completely restored, mice were subjected to IVGTT. Glucose tolerance was substantially improved but not normal (Figure 6C).

DISCUSSION

The principal finding of this study is that a combination of neogenesis and replication is capable of completely reconstituting a normal number of β -cells from a baseline in which there were very few preexisting β -cells. This is an important proof of principle that β -cell regeneration can be an effective therapy for diabetes, potentially even in settings where the number of β -cells is close to or at zero [12].

There are relatively few studies of β -cell regeneration in animals that underwent severe β -cell ablation [9] and none that we are aware of achieved complete β -cell reconstitution. Previously studied damage models induced β -cell neogenesis or β -cell replication, but not both. In our model, regeneration appears to have occurred in two distinct phases; an initial burst of β -cell neogenesis from α -cells followed by a wave of β -cell proliferation following Re-PDL. The initial burst of β -cell neogenesis in response to PDL plus alloxan recapitulated our previous study [4], but the

increased replication of islet endocrine cells following Re-PDL was surprising.

The initial impetus for trying to reverse PDL was to determine whether that would lead to a return to normoglycemia, and if so in what time frame. A previous study of severe β -cell ablation found that return to normoglycemia took 5-10 months, but it was unclear whether that was because continuing regeneration was required or because maturation was slow. Following Re-PDL, the blood glucose concentration began to drop immediately but slowly, along with the acquisition of markers of mature β -cells. Because of the wave of β -cell replication, we could not determine the extent to which maturation versus replication accounted for the return to normoglycemia.

In summary, beginning from a starting point of profound β -cell ablation, and in which the few surviving β -cells were immature, we have succeeded in achieving complete restoration of β -cell number and islet morphology and good restoration of β -cell function. This finding is encouraging and should provide further impetus for efforts to induce β -cell neogenesis and replication with pharmacologically relevant approaches that can be clinically translated.

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Figure 1. Reversal of PDL led to rapid recovery of the exocrine pancreas. Pancreases from mice that underwent PDL or PDL followed by reversal were immunostained for amylase (red) to visualize the acinar cells, and BrdU (green) to detect cells that had replicated. BrdU was administered continuously in the drinking water for 5-6 weeks beginning one day after Re-PDL or the analogous time in the controls. Re-PDL was done one week following PDL. (A-H) Following PDL, there was complete loss of exocrine tissue distal to the ligation (loss of amylase staining in B) compared with proximal to the ligation (normal amylase staining in A) and no evidence of recovery at later times (D). Following Re-PDL, there was rapid recovery of the acinar tissue (F, time course in I-L) and this was maintained at late points (H)(quantification in M of the area of longitudinal sections through the entire pancreas). Recovery was associated with an increase in replication (BrdU staining in F, H). Re-PDL early, n=5. Re-PDL late, n=7. PDL early, n=2. PDL late, n=2. Mice were sacrificed at days 35 (C, D) and 171 (G, H) following PDL. Scale bar=100 μ m.

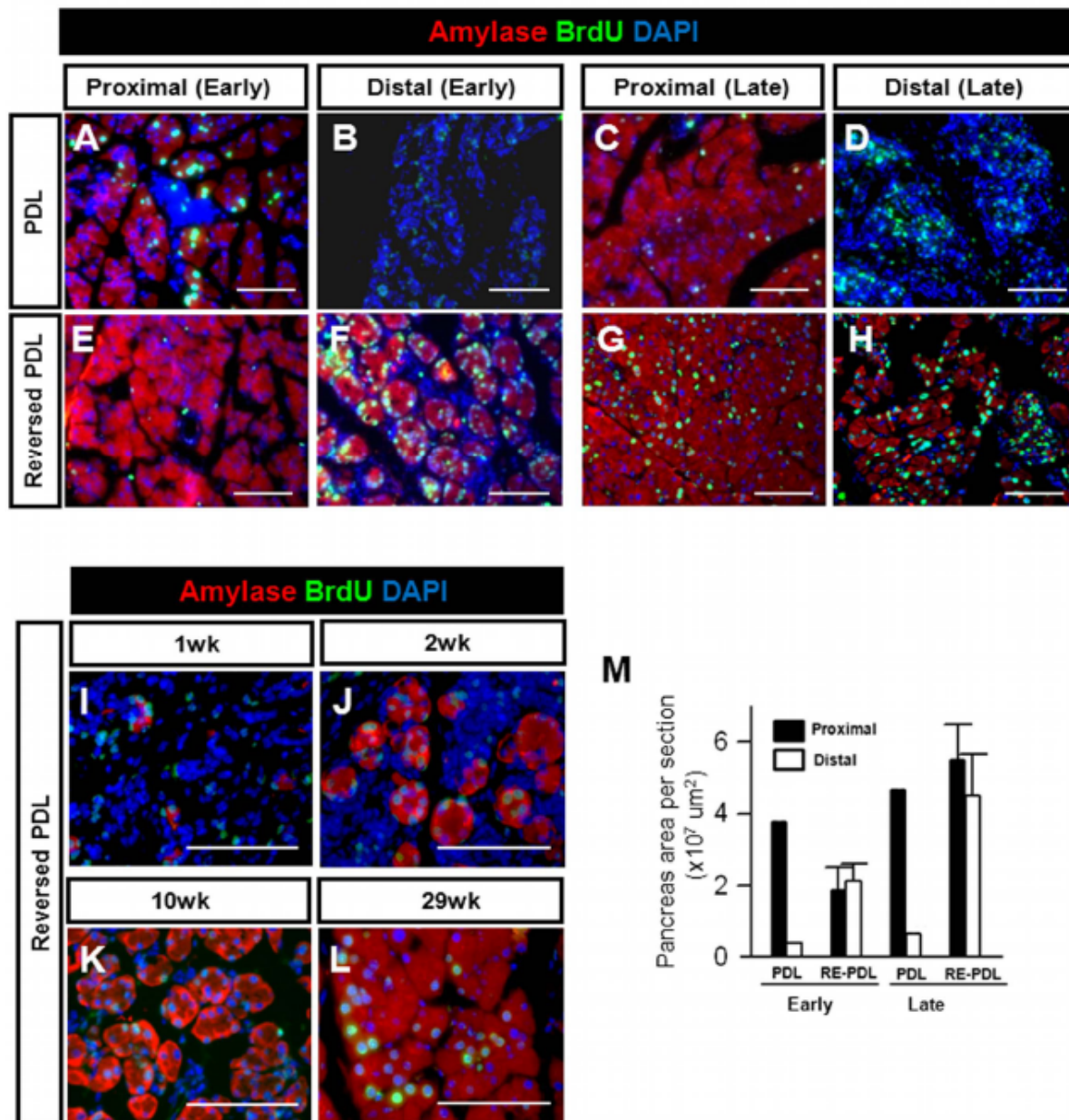


Figure 2. β -cell neogenesis and recovery of islet structure following Re-PDL. Shortly after PDL plus alloxan, there were few surviving β -cells proximal to the PDL (A, E) and this did not change at late times (C, G). There were no cells coexpressing insulin and glucagon. Note that yellow dots in A are autofluorescence. Distal to the PDL, insulin-glucagon co-positive cells were commonly found early (yellow cells in B, F), but were rare at late times following PDL (D, H). Re-PDL early, n=5. Re-PDL late, n=7. PDL early, n=2. PDL late, n=2. Mice were sacrificed at days 50 (C), 35 (D), and 171 (G, H) following PDL. Scale bar=100 μ m.

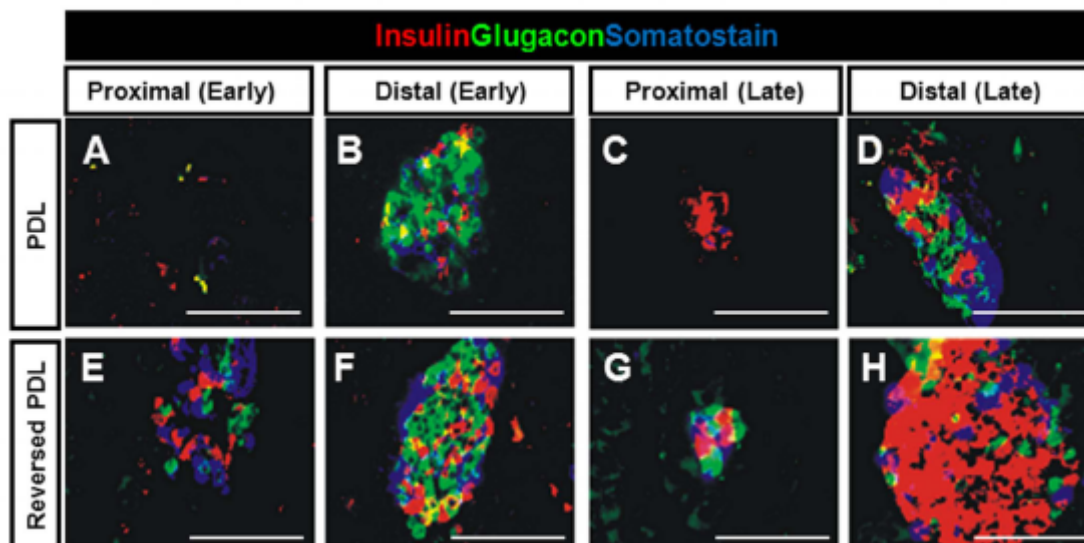


Figure 3. β -cell replication and number following alloxan plus PDL and Re-PDL. Following PDL, there was a small amount of replication in the exocrine pancreas and in the few remaining β -cells (A–D, low power and I–L, high power). Following Re-PDL, there was a large increase in replication in the exocrine pancreas distal but not proximal to the ligation (E–F, low power and M–P, high power). Notable particularly at later times following Re-PDL was a large increase in the number of replicating β -cells (compare H, P with D, L, quantified in S). This led to an increase in the number of insulin positive cells as measured by the number of insulin-positive cells (R) and the β -cell area (S). Note that the measurements in Q–S were made per pancreas section, each of which encompassed a $5\mu\text{M}$ longitudinal slice through the entire organ. This was done to control for the effect of PDL, which causes a dramatic drop in the pancreas area due to exocrine involution, but should have no effect on the absolute β -cell area on each section. Re-PDL early, $n=5$. Re-PDL late, $n=7$. PDL early, $n=2$. PDL late, $n=2$. Mice were sacrificed at days 35 (C, D, K, L) and 171 (G, H, O, P) following PDL. Scale bar= $100\mu\text{m}$.

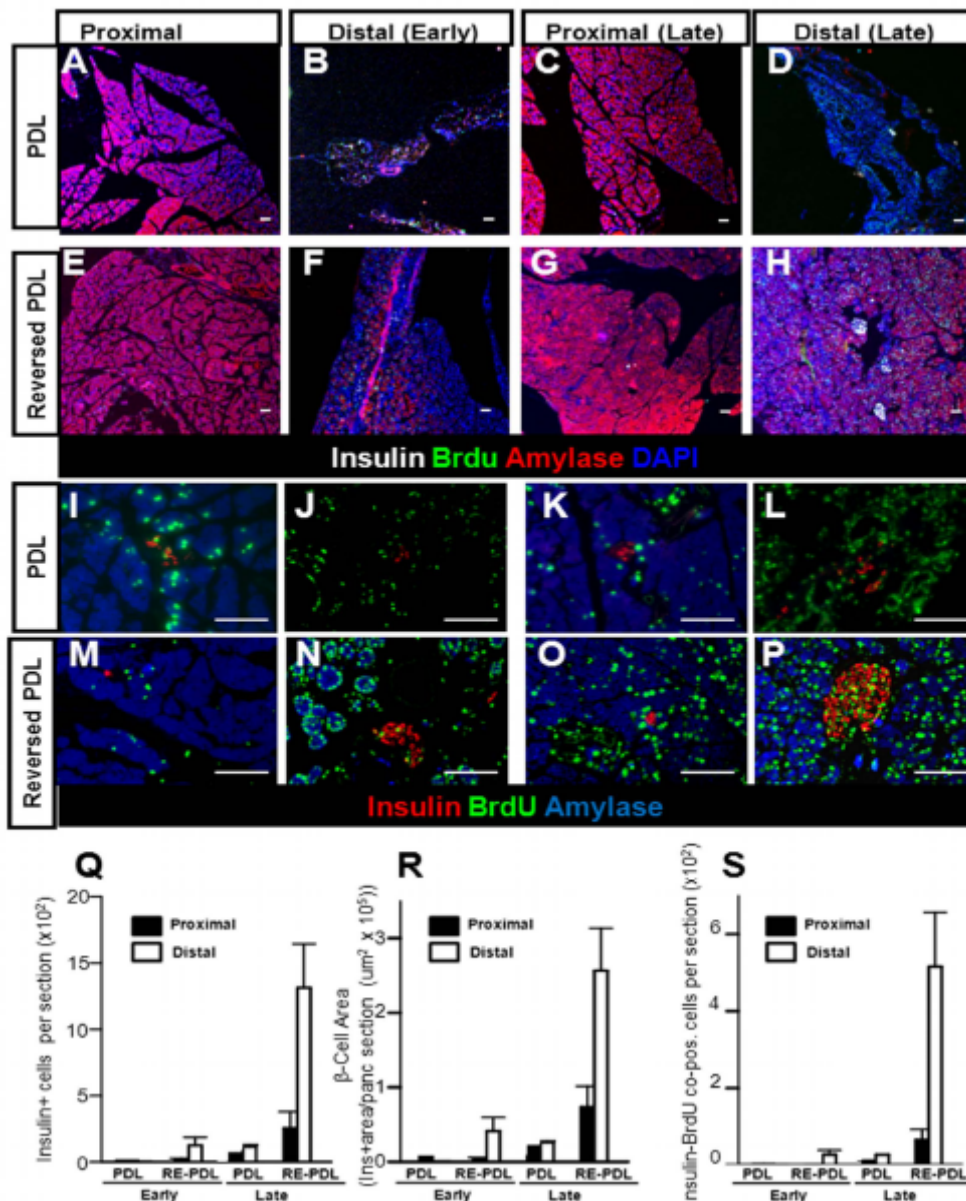


Figure 4. Islet structure and β -cell area returned to normal following Re-PDL. At the time of Re-PDL, one week after PDL, the islet structure was highly disorganized with a random mixture of α -, β -, and δ -cells (A). There was a gradual increase in the number of β -cells and a gradual normalization of the islet structure from weeks 2 (B), to 10 (C) to 30 (D). At late times following Re-PDL, the islet structure and area in the tail of the pancreas distal to the PDL was indistinguishable from normal (D, quantified in E). Note that the exocrine recovery at late times after Re-PDL allowed the use of pancreatic area rather than pancreas section for comparison of the β -cell area in the normal pancreas compared with the distal pancreas at later times following Re-PDL. Normal, n=4. Re-PDL, n=7. Scale bar= 100 μ m .

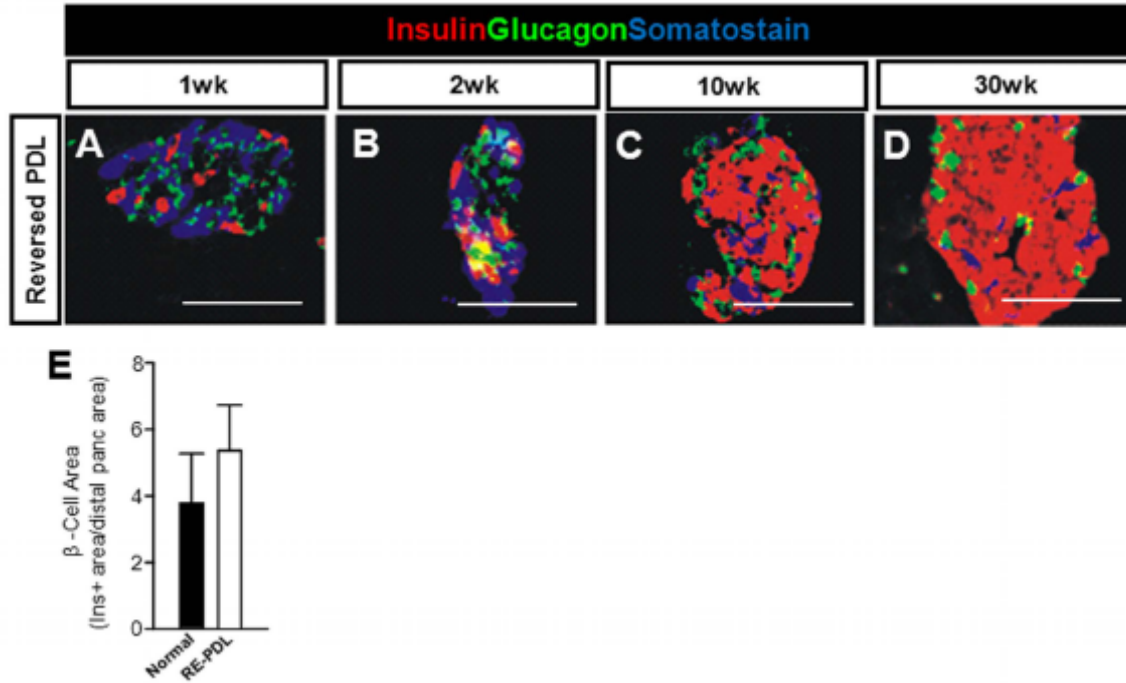


Figure 5. β -cell maturation following Re-PDL. Following PDL, the surviving β -cells (red) did not express GLUT2 (green) (A–D). GLUT2 was present late (G, H) but not early (E, F) following Re-PDL. Islet morphology was normal late following Re-PDL (H). Similar to GLUT2, MafA (green) was not expressed either early or late following PDL (I–L), but did become expressed in β -cells at late times following Re-PDL (arrows in P). Similar to GLUT2 and MafA, PDX1 (green) was not expressed either early or late following PDL (Q–T). PDX1 became expressed in β -cells at late times following Re-PDL (X). Re-PDL early, n=5. Re-PDL late, n=7. PDL early, n=2. PDL late, n=2. Mice were sacrificed at days 50 (C), 35 (D, K, L, S, T) and 218 (G), 210 (H) and 171 (O, P, W, X) following PDL. Scale bar=100 μ m.

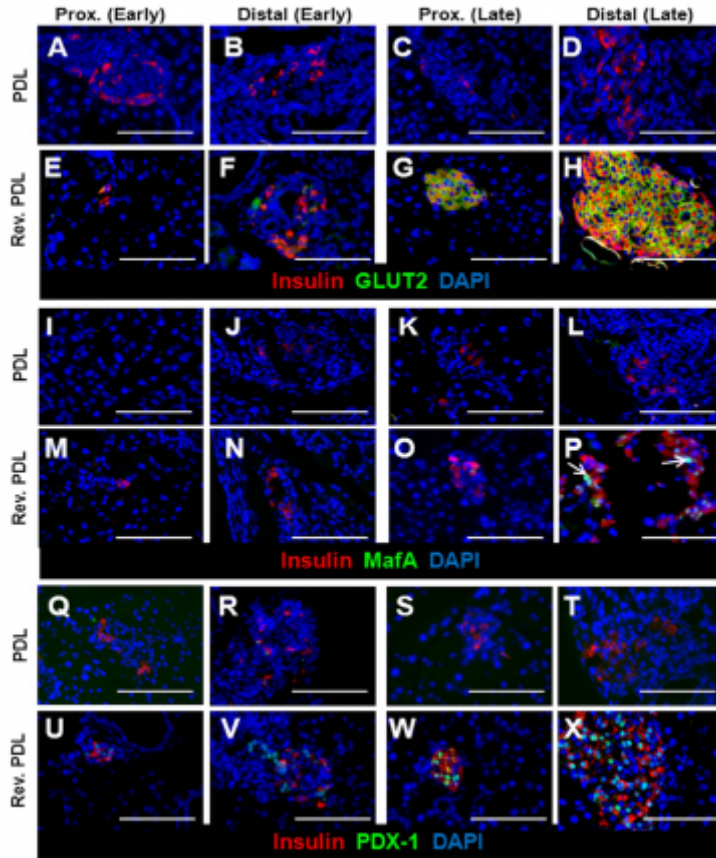


Figure 6. Glucose tolerance and survival following PDL and Re-PDL. Following PDL plus alloxan, the blood glucose concentration was greater than 500 mg/dL in all animals (A). The blood glucose began to drop immediately, but slowly, following Re-PDL (black line, n=25), except in one animal (green line) in which Re-PDL failed as determined by lack of exocrine recovery (Suppl. Figure 2). Control animals that did not undergo Re-PDL remained severely diabetic (red line, n=10). Kaplan-Meier survival plot of mice that underwent PDL with no reversal (red, n=10) or Re-PDL (black, n=26) demonstrating improved survival (B). IVGTT of normal (blue, n=5), alloxan-treated diabetic (red, n=2) or animals that underwent Re-PDL (black, n=3). IVGTT was done on animals that had reverted to normoglycemia and was performed 3-5 days prior to sacrifice (C).

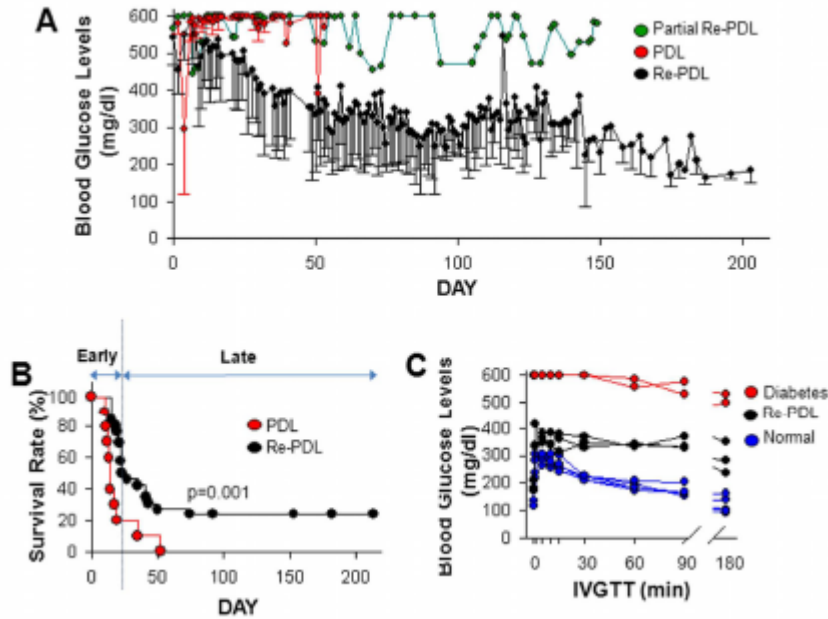


Table 1. Antibodies utilized in this study.

Antibody	Supplier	Dilution
Amylase (Rabbit)	Santa Cruz	1:400
Amylase (Goat)	Santa Cruz	1:400
BrdU (Mouse)	GE Health	1:400
Glucagon (Mouse, K79bB10)	Sigma	1:400
GLUT2 (Goat)	Santa Cruz	1:400
MafA (Rabbit)	Bethyl	1:100
Insulin (Guinea Pig)	USBIO	1:400
Insulin (Rabbit, H-86)	Santa Cruz	1:200
Pdx-1 (Goat)	Abcam	1:1500
Somatostatin (Goat, D-20)	Santa Cruz	1:200
Somatostatin (Rabbit)	Santa Cruz	1:200
Alexa 488	Invitrogen	1:400
Rodamin Red	Jackson Immuno Research	1:400
Daylight DY	Jackson Immuno Research	1:400